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Molecular Cloning and Sequence Analysis of the X-Prolyl Dipeptidyl Aminopeptidase Gene From *Lactococcus lactis* subsp. *cremoris*

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Lactococcus lactis subsp. *cremoris* P8-2-47 contains an X-prolyl dipeptidyl aminopeptidase (X-PDAP; EC 3.4.14.5). A mixed-oligonucleotide probe prepared on the basis of the N-terminal amino acid sequence of the purified protein was made and used to screen a partial chromosomal DNA bank in *Escherichia coli*. A partial *Xba*I fragment cloned in pUC18 specified X-PDAP activity in *E. coli* clones. The fragment was also able to confer X-PDAP activity on *Bacillus subtilis*. The fact that none of these organisms contain this enzymatic activity indicated that the structural gene for X-PDAP had been cloned. The cloned fragment fully restored X-PDAP activity in X-PDAP-deficient mutants of *L. lactis*. We have sequenced a 3.8-kb fragment that includes the X-PDAP gene and its expression signals. The X-PDAP gene, designated *pepXP*, comprises 2,289 nucleotide residues encoding a protein of 763 amino acids with a predicted molecular weight of 87,787. No homology was detected between *pepXP* and genes that had been previously sequenced. A second open reading frame, divergently transcribed, was present in the sequenced fragment; the function or relationship to *pepXP* of this open reading frame is unknown.

Lactococci are the most important components in starter cultures used in cheese manufacturing. These organisms are extremely fastidious and need an external source of amino acids. The concentrations of free amino acids and small peptides in milk are too low to support growth to high cell densities (27). For proper growth, lactococci are dependent on a proteolytic system to degrade milk proteins. This system is composed of a cell envelope-associated proteinase and a number of different peptidases (for reviews, see references 20 and 36) which act in concert to hydrolyze milk proteins to amino acids and transportable peptides (30). The proteinase is the first enzyme active in casein degradation (for a review, see reference 19), releasing peptides which can be used as substrates for intra- or extracellular peptidases.

As the casein-derived products generated by the proteinase are rich in proline and the peptides are too large to enter the cells (28) and as some of the essential amino acids are present in these proline-rich peptides, lactic acid bacteria need proline-specific peptidases, even if proline itself is not essential for growth. Most of the peptidases isolated from lactic acid bacteria and characterized are not capable of releasing N-terminal or penultimate proline residues. An X-prolyl dipeptidyl aminopeptidase (X-PDAP) activity has been demonstrated in a wide range of lactic acid bacteria (5). This enzyme is capable of releasing dipeptides from oligopeptides containing proline at the penultimate position. X-PDAPs have recently been purified from *Lactobacillus casei* and *Streptococcus thermophilus* (25), *Lactococcus lactis* subsp. *cremoris* P8-2-47 (16), *L. lactis* subsp. *lactis* NCDO 763 (41), *L. helveticus* (15), and *L. delbrueckii* subsp. *bulgaricus* (3) and characterized. The pH and temperature optima of these enzymes are around 7.0 and in the range of

45 to 50°C, respectively. These enzymes range from 72,000 to 90,000 daltons. Most of the enzymes are inhibited by phenylmethylsulfonyl fluoride and diisopropyl fluorophosphate but not by EDTA, probably indicating that they belong to the class of serine proteinases. The location of X-PDAPs is still a matter of dispute; most reports assume that they are intracellular enzymes, but an extracellular location has also been postulated (16).

In this paper, we report on the cloning, expression, and sequence of the X-PDAP gene (*pepXP*) from *L. lactis* subsp. *cremoris* P8-2-47. Independently, a highly similar gene from *L. lactis* subsp. *lactis* NCDO 763 was isolated and characterized (29a).

MATERIALS AND METHODS

Bacterial strains, plasmids, and media. The bacterial strains and plasmids used are listed in Table 1. *L. lactis* was grown in M17 medium (Difco, East Molesey, United Kingdom) with glucose or lactose at 30°C. *Escherichia coli* and *Bacillus subtilis* were grown in TY broth (Difco, Detroit, Mich.) at 37°C with shaking. When needed, ampicillin (75 µg/ml for *E. coli*), erythromycin (100 µg/ml for *E. coli* and 5 µg/ml for *L. lactis* and *B. subtilis*), or chloramphenicol (10 µg/ml for *E. coli*) was added to the media. 5-Bromo-4-chloro-3-indolyl-β-D-galactopyranoside (Sigma Chemical Co., St. Louis, Mo.) and isopropyl-β-D-thiogalactopyranoside (Sigma) were used at 0.004% (wt/vol) and 0.5 mM, respectively.

Southern hybridization. DNA was transferred to Gene Screen Plus membranes (Dupont NEN, Boston, Mass.) as described by Maniatis et al. (24). Hybridizations were performed at 35 to 40°C in the presence of oligonucleotide probes end labeled with [γ -³²P]ATP (Amersham, Buckinghamshire, United Kingdom) by use of T4 polynucleotide kinase by the protocol of Duby (8). Nonradioactive DNA probes were labeled with digoxigenin-dUTP by using a nonradioactive labeling and detection kit (Boehringer,

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant genotype or phenotype ^a	Reference or source
Strains		
<i>E. coli</i> JM103		40
<i>B. subtilis</i> 6GM		12
<i>L. lactis</i> subsp. <i>lactis</i>		
IL1403	Plasmid-free X-PDAP ⁺ strain	6
MG1363	Plasmid-free X-PDAP ⁺ strain	10
BM10 to BM12	X-PDAP ⁻ mutants of IL1403	This work
BM20 and BM21	X-PDAP ⁻ mutants of MG1363	This work
<i>L. lactis</i> subsp. <i>cremoris</i>		
P8-2-47	X-PDAP ⁺ strain	2
BM30 to BM35	X-PDAP ⁻ mutants of P8-2-47	This work
Plasmids		
pUC18	Ap ^r , 2.68 kb, <i>lacZ</i> , pBR322 derivative	40
pUC19	Ap ^r , 2.68 kb, <i>lacZ</i> , pBR322 derivative	40
pGKV2	Em ^r Cm ^r , 4.4 kb	38
pHPS9	Em ^r Cm ^r , 5.7 kb, <i>lacZ</i>	12
pBM330	5.4-kb partial <i>Xba</i> I fragment in pUC18	This work
pBM329	5.4-kb partial <i>Xba</i> I fragment in pGKV2	This work
pBM3330	3.8-kb partial <i>Pvu</i> II- <i>Xba</i> I fragment in pHPS9	This work

^a X-PDAP⁺, X-PDAP positive; X-PDAP⁻, X-PDAP negative; Ap^r, Em^r, and Cm^r, resistance to ampicillin, erythromycin, and chloramphenicol, respectively.

Mannheim, Germany); hybridization and immunological detection were done as recommended by the supplier.

DNA manipulations and sequencing. Chromosomal DNA was isolated from *L. lactis* subsp. *cremoris* P8-2-47 by the method of Leenhouts et al. (22). Plasmids from *E. coli* were isolated as described by Birnboim and Doly (4). With some modifications (37), the same method was used for the preparation of plasmid DNA from *L. lactis* and *B. subtilis*. Restriction endonucleases and T4 DNA ligase were purchased from Boehringer and used as recommended by the supplier. General procedures for DNA manipulations were essentially those described by Maniatis et al. (24). *E. coli* and *B. subtilis* cells were made competent and transformed by the protocols of Hanahan (13) and Hardy (14), respectively. Transformation of *L. lactis* was done by electroporation with a Gene Pulser (Bio-Rad Laboratories, Richmond, Calif.) by the method of Leenhouts et al. (22). Subfragments of the 3.8-kb *Pvu*II-*Xba*I fragment of pBM330 were cloned in pUC18 and pUC19 for sequencing. The nucleotide sequence determination was carried out by the dideoxy method of Sanger et al. (33) with 5'-[α -³⁵S]dATP (Amersham). DNA and amino acid sequence analyses were performed with the Microgenie sequence analysis software package (Beckman, Palo Alto, Calif.).

Oligonucleotide synthesis. The oligonucleotide probes and primers were synthesized on a Millipore Cyclone DNA synthesizer (Millipore GmbH, Eschborn, Germany) or in an Applied Biosystems synthesizer (Applied Biosystems Inc., Foster City, Calif.) by the β -cyanoethyl phosphoramidite procedure (34).

X-PDAP purification and N-terminal amino acid sequence determination. X-PDAP was purified by the method of Kiefer-Partsch et al. (16). The protein was electroblotted from the gel onto a polyvinylidene difluoride membrane in accordance with the instructions of the manufacturer (Immobilon; Millipore, Bedford, Mass.). Purified X-PDAP was analyzed with a Knauer 810 protein sequencer and a Knauer high-pressure liquid chromatography analyzer (Knauer, Berlin, Germany) equipped with an Applied Biosystems PTH-C column (220 by 2.1 mm).

Mutagenesis and plate assay for X-PDAP activity. Methyl-

methane sulfonate (Eastman Kodak, Rochester, N.Y.) mutagenesis of *L. lactis* was performed on a mid-log-phase culture in M17 medium as described by Anderson and McKay (1). A survival percentage of 10% was chosen. Colonies were screened for X-PDAP activity by a plate staining procedure described by Miller and Mackinnon (26). L-Glycyl-L-prolyl- β -naphthylamide and L-alanyl-L-prolyl-p-nitroanilide were purchased from Bachem (Budendorf, Switzerland), and fast garnet GBC was purchased from Sigma.

Enzyme activity in crude cell extracts. Crude cell extracts were prepared from late-log-phase or stationary-phase M17 broth cultures. Cells were harvested by centrifugation, washed twice in 0.1 M phosphate buffer (pH 7.0), and suspended in 0.05 M Tris hydrochloride buffer (pH 7.5). Cells were disrupted by sonication (Soniprep 150; MSE Scientific Instruments, Crawley, United Kingdom), and the resulting suspension was centrifuged at 80,000 $\times g$ for 20 min in a Beckman L8-80 centrifuge to remove cell debris. X-PDAP activity in the supernatant was measured as described by Miller and Mackinnon (26) with L-alanyl-L-prolyl-p-nitroanilide as the substrate. Activities were expressed as units per milligram of protein present in the crude cell extracts. Protein concentrations were measured by the method of Lowry et al. (23).

Nucleotide sequence accession number. The GenBank and EMBL nucleotide sequence accession number is M58315.

RESULTS

Cloning of the X-PDAP determinant. After purification of X-PDAP of *L. lactis* subsp. *cremoris* P8-2-47, approximately 200 pmol of protein was used to determine the 25 N-terminal amino acids of the enzyme. To clone the structural gene of X-PDAP, we synthesized an oligonucleotide probe on the basis of the sequence of amino acids 10 to 15 of the sequenced stretch (Fig. 1). Chromosomal DNA of *L. lactis* subsp. *cremoris* P8-2-47 was digested with several restriction enzymes; after electrophoresis, the DNA was transferred to Gene Screen Plus membranes. The ³²P-labeled oligonucleotide probe was used for hybridization to the DNA fragments under various conditions. The optimum condition for hybrid-

Met - Arg - Phe - Asn - His - Phe - Ser - Ile - Val - Asp -
 Lys - Asn - Phe - Asp - Glu - Gln - Leu - Ala - Glu - Leu -
 Asp - Gln - Leu - Gly - Phe

FIG. 1. N-terminal amino acid sequence of X-PDAP of *L. lactis* subsp. *cremoris* P8-2-47. The sequence chosen for synthesizing the 17-bp oligonucleotide probe is underlined. The resulting probe was 5' GAT(C)-AAG(A)-AAT(C)-TTT(C)-GAT(C)-GA 3'.

ization was overnight at 37°C. Various washing procedures were tested; ultimately, washing of the filters was performed as follows: twice for 10 min with 2× SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 0.1% sodium dodecyl sulfate and 0.1 mM PP_i at room temperature and twice for 20 min with the same solution at 35°C. Under these conditions, the probe hybridized with several bands (Fig. 2). The DNA of the chromosomal digests corresponding to the most intense bands of hybridization (Fig. 2) was isolated from the agarose gels by electroelution. The restriction enzyme fragments were ligated to pUC18 cut with the corresponding enzymes, and the ligation mixtures were used to transform competent *E. coli* JM103 cells. Transformants were screened for hybridization with the oligonucleotide probe. As *E. coli* does not have X-PDAP activity, the transformants could also be directly screened for the pro-

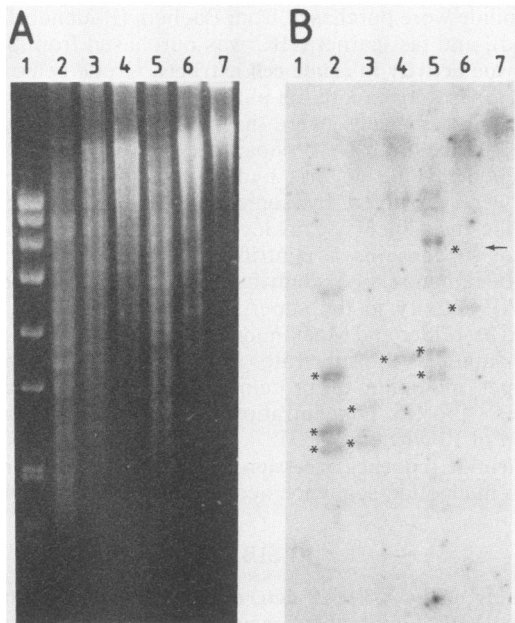


FIG. 2. Southern hybridization of DNA of strain P8-2-47. (A) DNA samples on an 0.8% agarose gel. Lanes: 1, marker DNA (phage SPP1 DNA digested with *EcoRI*; the sizes of the visible bands are 8.54, 7.42, 6.15, 4.90, 3.59, 2.84, 1.97, 1.91, 1.56, 1.45, and 1.16 kb); 2 to 7, total DNA of strain P8-2-47 cleaved with *HindIII*, *PvuII*, *BclI*, *EcoRI*, and *XbaI* and undigested, respectively. (B) Autoradiogram of a Southern blot of the gel shown in panel A hybridized with the ³²P-labeled mixed-oligonucleotide probe. The restriction fragments of the bands marked with asterisks were cloned in pUC18. The hybridizing *XbaI* fragment producing X-PDAP-positive transformants is indicated by an arrow.

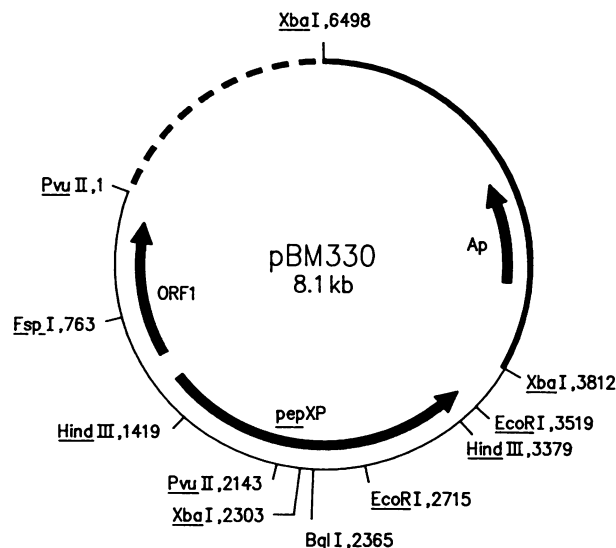


FIG. 3. Schematic representation of plasmid pBM330. Arrows represent the length and orientation of the *pepXP* gene, ORF1, and the Ap^r gene. The heavy solid line represents pUC18. The broken line represents an unsequenced 1.5-kb part of the 5.3-kb *XbaI* fragment. The part of the plasmid represented by the thin line was completely sequenced. Only in the sequenced fragment are the restriction endonuclease sites indicated.

duction of X-PDAP by the plate assay technique described in Materials and Methods.

In this way, three X-PDAP-positive clones were obtained from the transformants carrying DNA from the largest hybridizing *XbaI* band. All three clones carried an insert of 5.3 kb which, upon digestion with *XbaI*, was cleaved into two pieces. Apparently, in all three cases a partial *XbaI* fragment had been cloned. The partial *XbaI* fragment from the three positive clones was found in the two possible orientations, suggesting that the gene was expressed from its own promoter. The same results were obtained in consecutive transfers of the insert to several other vectors (data not shown). The expression of X-PDAP in *E. coli* strongly suggested that we had cloned the structural gene for X-PDAP. The restriction map of pBM330, one of the plasmids containing the 5.3-kb partial *XbaI* fragment, is shown in Fig. 3. Southern blot analyses of restriction enzyme digests and subsequent hybridizations showed that the mixed-oligonucleotide probe hybridized exclusively with the *PvuII* (position 1)-*HindIII* (position 1419) fragment of pBM330, locating the 5' part of the X-PDAP gene on this DNA fragment (data not shown). The smallest recombinant plasmid with X-PDAP activity, designated pBM3330, carried a 3.8-kb *PvuII* (position 1)-*XbaI* (position 3812) fragment inserted in pHPS9 (12).

Construction of pBM329 and X-PDAP expression in *B. subtilis*. The smallest *XbaI* fragment of pGKV2, a derivative of the broad-host-range plasmid pWV01 (38), which replicates in lactococci as well as in *E. coli* and *B. subtilis*, was replaced by the 5.3-kb partial *XbaI* fragment of pBM330. An X-PDAP-producing clone was recovered in *E. coli*, and the new construct (pBM329) was introduced into *B. subtilis* by competent cell transformation. All the transformants acquired X-PDAP activity, an activity not normally present in this bacterium. This result supports our conclusion that the structural gene for X-PDAP had been cloned.

Complementation in X-PDAP-negative mutants of *L. lactis*. Wild-type colonies of *L. lactis* on M17 agar plates exposed to a mixture containing L-glycyl-L-prolyl- β -naphthylamide and the diazonium salt fast garnet GBC turn red in 10 to 20 min. To obtain X-PDAP-deficient *L. lactis* mutants, we stained colonies from a culture mutagenized with methylmethane sulfonate in the same way. Colonies which stained only to a pale orange color were obtained at a frequency of approximately 10^{-4} . Several such colonies were purified and retested for their altered staining properties. In this way, stable mutants were obtained from *L. lactis* subsp. *lactis* MG1363 and IL1403 as well as from *L. lactis* subsp. *cremoris* P8-2-47. X-PDAP activity in these mutants ranged from 0 to 25% that in the parental strains (0.172 ± 0.004 U/mg of protein per min). pBM329 was introduced into *L. lactis* mutants by electroporation, and all mutants recovered the original X-PDAP-proficient phenotype. The specific X-PDAP activities conferred by plasmid pBM329 on the X-PDAP-negative mutants were compared with those of the parental strains. Measurements were done with log-phase cells in all cases. Activities were not significantly different between the mutants carrying pBM329 and the wild-type strains without this plasmid (0.168 ± 0.004 U/mg of protein per min), despite the fact that the X-PDAP determinant was cloned on a plasmid present in several copies per chromosome (between five and eight) (38).

DNA sequence analysis. The 3.8-kb *Pvu*II (position 1)-*Xba*I (position 3812) fragment of pBM330 was subcloned in *E. coli* on pUC18 and pUC19, and both strands were sequenced in a cascade-sequencing strategy with synthetic primers. These primers were also used to confirm the sequence data around the restriction enzyme sites used for subcloning. The sequence is presented in Fig. 4. Analysis of the sequenced region revealed the presence of two complete open reading frames (ORFs). The largest of these starts, with an ATG start codon at position 1213 and the first stop codon (TAA), is located at position 3502, giving it a total length of 2,289 bp or 763 coding triplets. It has the potential to specify a protein of 87,787 daltons. The N-terminal 25 amino acids of the protein specified by this ORF, underlined in the figure, are the same as those determined in purified X-PDAP. On the basis of this result, we conclude that this ORF is the gene for X-PDAP (*pepXP*) of *L. lactis* subsp. *cremoris* P8-2-47. The molecular mass of X-PDAP deduced from the nucleotide sequence (88 kDa) agrees well with that of the purified enzyme estimated from sodium dodecyl sulfate-polyacrylamide gel electrophoresis for the monomeric form of the enzyme (89 kDa) (16). No putative signal peptide or obvious membrane-spanning domains were found in X-PDAP. Upstream of the start codon, near nucleotide 1203, a strong Shine-Dalgarno sequence (GGAGG) similar to those reported for *B. subtilis* (11) and *L. lactis* (7, 39) is present. Around position 1165 a potential promoter region is present. The -10 sequence (TATAAT) is identical to the -10 region of consensus *E. coli* and vegetative *B. subtilis* promoters (32). The corresponding putative -35 sequence (CTGATT) is located around position 1150. However, alternative sequences that could function as *Lactococcus* promoters are also present. No clear terminatorlike structure was observed downstream of the stop codon of the *pepXP* gene.

The second ORF, named ORF1, in the opposite orientation relative to *pepXP*, starts at position 1041 with an ATG start codon and ends at position 175. The total length is 867 bp or 289 coding triplets. The estimated molecular mass of the inferred protein is around 31,000 daltons. Upstream of the ATG start codon, a Shine-Dalgarno sequence (GGAGG)

is present at position 1055, as are possible -10 (TATAAT) and -35 (TTGAAG) promoter sequences. Hydropathy analysis of the amino acid sequence revealed that the ORF1 product is very hydrophobic, with five or possibly six membrane-spanning domains. Downstream of the ORF1 stop codon, a region of dyad symmetry located between nucleotides 132 and 173 is capable of forming a stem loop of 19 bp (containing three G · C pairs and no mismatches) and could act as a transcription terminator, with a free energy (ΔG) of -23.0 Kcal [ca. -96.0 kJ/mol] (32). The region containing the putative promoter sequences for ORF1 and *pepXP* is AT rich, with a percentage of A · T pairs of 78. Such AT-rich sequences appear to be characteristic for lactococcal promoter regions (39).

Codon usage and G+C content. Codon usage in *L. lactis* ORF1 and *pepXP* was compared with that in *E. coli* and *B. subtilis* genes. For the amino acids Phe, Ile, Lys, and Leu, *B. subtilis* and *L. lactis* have the same preferred codons, whereas *E. coli* preferentially uses other ones. Also, rare codons in genes of gram-negative organisms (11), e.g., TTA, GTA, ACA, AAA, CGA, AGA, and GGA, are commonly used in *L. lactis pepXP* and ORF1. Furthermore, there is a strong bias against G and C, especially in the third position in both *pepXP* and ORF1, which is at variance with the plasmid-encoded lactococcal proteinase gene (18). Codon usage in *L. lactis* differs from that in both *B. subtilis* and *E. coli* in its distribution of codons specifying Pro, Ser, and Val.

The G+C content of the sequenced DNA fragment was 37.5%, a value which agrees well with the mean G+C values of four different lactococcal strains (17).

Homology comparison. None of the proteins present in the SWPROT Swiss protein bank on April 1990 showed significant amino acid sequence similarity to the *L. lactis pepXP* gene product. No similarities were found with serine proteinases, despite the fact that the X-PDAP enzyme has been classified as a serine peptidase (16, 41). Neither was homology found with the product of a sequenced X-PDAP gene product from *Saccharomyces cerevisiae* (31). The putative ORF1 protein showed considerable amino acid similarity to the glycerol facilitator protein from *E. coli* (29; 26.5% identity in a stretch of 151 amino acids) and nodulin-26 from *Glycine max* (soybean) (9; 26.9% identity in a sequence of 108 amino acids). Both proteins are believed to be integral transmembrane proteins.

DISCUSSION

X-PDAP from *L. lactis* subsp. *cremoris* P8-2-47 is the first lactococcal peptidase for which a primary structure has been determined. The gene was present on a partial *Xba*I fragment produced as the result of incomplete digestion of chromosomal DNA with *Xba*I. Expression of the gene in *E. coli* and *B. subtilis*, both devoid of X-PDAP activity, strongly suggested that the fragment encodes the X-PDAP structural gene. Moreover, the 5.3-kb fragment containing the sequenced region fully restored the enzymatic deficiency in *L. lactis* X-PDAP-deficient mutants. Cloning of the determinant in a plasmid vector with a copy number of approximately six in lactococci did not result in an increase in X-PDAP activity, suggesting that the enzyme is subject to some kind of regulation.

Sequence analysis of the region specifying X-PDAP revealed the presence of two complete ORFs, one of which could be identified unequivocally as the X-PDAP gene (*pepXP*), because the first 25 amino acids of this ORF were exactly the same as those determined from the purified

Pvu II
 CAGCTGGTTAACTTATGGACTGATGAGTCTTGCTTACTGGGCTCTATGCTAAGTATCGTAATTTTTATCTCAATATGAGTGCTCCATTTCTTTGGAACTTTTGGACTAGTCATTGCG 120
 T
 ACTTATGGTCAAAATAAAAAAATCTGACAACTCTGTCAGTAATTTTTATTAAAGATAAATCATTTTAAATAATGCAACGGCTGCAAGTGATGCAAGAAATGGTGCCAAAACCTGGAAAC 240
 W A Y W W K S S G K A E G L V S K P L L S H V L R P G F D R A P N L G P G T F G
 CCCTGCATACCACCATTTAGATGAACCTTTAGCTTACCCAAAACGATTTTGGTAAGAGACTGTGAACCAACGTTGACCAAGTCAACGCGCGGATTCAAACAGGACCTGTAGTCC 360
 G L A V V L G M V L P G L F L H A I N K S A S A G S A Q V W I Q N M V D S S S V
 ACCAAGTGCAACTACGAGTCCCAATAACAAGGAATCTAGGAACAAGTGAGCAATCATTTTGAAGCTGCTGATGCTTGAACCAAAATTTGATTCAACGCTGTGATGAAGAALAC 480
 D A G Q G K L Y N T H W T I S Q S G F F I N T A A V A G F F L V P S G L F E N I
 ATCTGCTCTTACCTTTCAAGTAGTTGTCATCCAAGTAATGATTGACTACCAAGAAGATATTTGTCGAGCAACCGCACCGAAGAACAATACAAATGAACCTAAGAACTCATTCAA 600
 F G N I T A G L R T K E S N D D V N D I T S P T G L I A N P N Q T R K L Y Y P R Y
 GAAACCATTAATGGTTGCTCCCAAGAGTTTTTTCATGTTATCATCAACGTTGTCGATAGTTGAGAAAGTTCCAAGAATAGCATTGTGATTGTGTTTAAAGATAGTAGGACGATA 720
 V M V I L L Q G F H A G L V Q A I I Y Q A V H A W P F L G S A A L G L T F A P N
 AACCATAACGATTAATAATTTGTCACCAATCGCACCTAATCTTGGCAATAATGATTGAGCAACGTTGCCAAGGAAAAGTCCAGGCTGCAAGTCCAGTGTAAGCTGGGTT 840
 I Q S T I N G F A V A P L M V G L G Y G W G I I M W S Q A H A K T G K L Y Y P R Y
 GATTGTGAAAGTAATATACCAAAAGCAATGTCGGCAAGTTAGCGCAAGTAAACCAACCAATATCATCAAGATTGTGCGTGAGCTTTGTACCTTTAAGTTCAACGTTAGC 960
 V A G N G M I I L L A T G V F E T I Y K V T W T V D M
 AACCGCACCATTAACCAATAATAGTAGGCGAGTTCCCAAAATTCAGTGATATATTTCACTGTCATTAACATCCATTTTATGGAACCTCCAAAATTTGATTTTAATATAATA 1080
 RBS
 ACCTCAGCTATTATACCAAGAAAGCAGCTTGGCTTCAAAATATTCATTGAAAATAGCGCGCTTTAGTCTGTTAATCACTGAAAATATGCTATAATTAAAAGAAATATCATGTTTATTAC 1200
 -10 -35 -35 -10
 RBS
 GGAGGATTAAAATGCGCTTAAACATTTTCAATTGTTGACAAAAATTTGATGAACAATAGCAGAATCTGATCAGCTTGGTTTTCGTTGGTCTGTTTTTGGGATGAAAAGAAAATC 1320
 M R F N H F S I V D K N F D E Q L A E L D Q L G F R W S V P W D E K K I
 TTAAGATTTTCTCATTAAGTCTAGTGATAGCGGATCTACAAGCACTGCTGAATAGATGTCATGTAATTTTAAAATCCCTATAGAATTAGATTGGGAAATTTCTGGAAAT 1440
 L K D P L I Q S P S D M T D L Q A T A E L D V I E F L K S S I E L D W E I F W N
 ATTGCTTGGCACTTCTGATTGTTGACCAAACTTTGATTGAGATTGGCAAGCTTTGAGTATGCAAAAATAGCAACCTCCCTCAATCGAAGCAGAAATGACCACAGAAATATC 1560
 I A L Q L D F V P N F D F I G K A F E Y A K N S N L P Q I E A E M T T E N I
 ATTTCGCTTTCTACTACCTCTTGTACGCGTGAAGAAATGGAATGATTGTTGTAACATTGGGTATCAGAGGACTTCTCTTGTAGATAATCATATTCTTTTCAATGACAAA 1680
 I S A F Y Y L L C T R R K N G M I L V E H W V S E G L L P L D N H Y H F F N D K
 TCGTTGGCAACTTGTATTCTTCACTTCTGAACGTGAAGTCTTGGGTTGAAGTCCAGTCGATAGTGAAACGCGGTGAAAATGATTGATAAAAAATCAATATCATGTCACAAA 1800
 S L A T F D S L L E R E S L W V E S P V D S E Q R G E N D L I K I Q I I R P K
 AGTACGAAAAACTTCCAGTGGTAAGTACGCGGAGTCTTATCATTTAGGCAATTAATGATAAAGCAATGATTAGCTTACACGATATGAATGTAGAACTTGAAGAAAAACCATGAC 1920
 S T E K L P V V M T A S P Y H L G I N D K A N D L A L H D M N V E L E E K T S H
 GAAATTCATGTGAACAAAGCTCCCGCAAAATATCAGCGAAAGCAAGGAATCCGATTGTTGACAAAGCTCTTATCGTTTACTCATGTTGGACTTATTCTTTAAATGACTAT 2040
 E I H V E T G K L S A K A K E L P I V D K A P Y R F T H G W T Y S L N D Y
 TTCTTAACGAGGATTGCTCTATCTATGTTGGCGGTGTTGGGACGCTTCTCTGACGCTTCAAACTCAGGTGATTACCAACAAATTTATAGTATGACAGCTGTCATTGACTGG 2160
 F L T R G P A S I Y V A G V G T R S S D G F Q T S G D Y Q Q I Y S H T A V I D W
 TIGAATGGTCGAGCTCGTCTTACACTTCTCTGTAAGAACACATGAAATTAAGCCCTTGTGGGCAATGCTAAGTGGCTATGACTGGAATACTTTATGTTGGAACTGCTTACGA 2280
 L N G R A K A Y T F S R K A T H E I K A C S W A N G K V A H T G K S Y L G T M A Y G
 GCGCTACTACTGGAGTTGAAGCTCTAGAGTTATCTTAGCAGAAGCTGAATTTCTTCTGTTGATAATTAATCCGTAAGAAATGCGCTTCTGCTGCGGCTTCTGCTGGTGA 2400
 A A T T G V E G L E V I L A E A G I S S W Y N Y Y R E N G L V R S P G G F P G E
 GATTAGATGTTCTGCTGCTCACTTATTCACGTAATCTTGATGGTGCTGATTCTTAAAGGTAATGCTGAATACGAAAACGATTGGCAGAAATGACTGCTTTAGACGCAAA 2520
 D L D V S L A L D G A L E Y S D K A R V K D F L K K Y S K D P N V F L S A Q I
 TCTGGTGATTACAATCAATTTGGCATGACGGAATTAATCTGATAAATAGTACAAAGTTAAGGCTGATGCTTAAATGCTTCAATGCTGACTACAAGATTGGAATGTTACACGAGCAAGCC 2640
 S G D Y N Q F W H D R N Y L I N T D K V K A D V L I V H G L Q D W N V T P E Q A
 TATAATTTCTGGAAGCTCTGCCAAGGTCATGCTAAACATGCCCTTTTACACGCTGGGCTCATATTATATGAATTTCTGGCAGTCCATTGATTTCTGGAACGATTAATGCTTAC 2760
 N P F L G K T A V S F A Q F D N H Y D E T P K K Y S K D P N V F L S A Q I
 TTTGTGCAAAATTAAGTATGATGATTTAAATTTAAATCTCCACCTGTTATTTTACAGGAAAATCTAAAGACCAAGTATGACGATGATGAATGATTGCTGCAATACCCAAATC 2880
 F V A K L L D R D L N L N L P P V I L Q E N S K D Q V W T M H N D P G A N T Q I
 AAACCTCCCTCTGGTAAACGCGCTTCTTTGCTCAATTCGACAAATCATATGACGATGAGACTTTCAAAAGTATTCTAAAGATTTCAATGCTTTTAAAGAGACTTATTGTAAC 3000
 K L P L G K T A V S F A Q F D N H Y D E T P K K Y S K D P N V F L S A Q I
 AAAGCCAATGAAGCTGTCATTGATTAGAACTTCCGTCAATGCTGACAATTAATGGCCAGTTGAGCTTGAACCTCAGACTAAAATTAATGACAGAAAGGCTTCTGCTGCTCAAAAT 3120
 K A N E A V I D L E L P S M L T I N G P V E L E L R L K L N D T K G F L S A Q I
 CTGATTGTTGGTCAGAAAAACGTTTGGAGATAAAGCCAGAGTGAAGATTTTAAAGTCTGACCGTGGGCGAAATTCATGTTAGACGACTTAGTTGAACCTCCACTGTTGAGAGC 3240
 L D F G Q K K R L E D K A R V K D F L K K Y S K D P N V F L S A Q I
 CCTATCAGTTAATCACTAAAGGCTTACTAATCTCCAAATCAAAATTTACTGACAGTCAGTGATTAAAGGCTGACGAATGGTTTACAATAAAATTTGAACCTACAGCAACCATTTAT 3360
 P Y Q L I T K G F T N L Q N Q N L L T V S D L K A D E W F T I K F E L Q P T I Y
 CATTTGAAAAAGCTGACAAAGCTTGGGTCATTCTCTATAGTACTGACTTTGAACATACGCTTCTGATATCGTAANGTAACCTACGAGATTGATTTATCTCAATCTAAACCTATTAT 3480
 H L E K I L Y S T D F E H T V D R N R K V T Y E I D L S Q S K L I I
 CCTATTGAAGTGTAAGAAATTAATATGGCAATTAATGGAATTTCTGGAACCTCTTACAACACAGTTGAACGTTCTCTTTTGTATGGAACAAAGTAAGCTATACAGCTCAATCTTTTAT 3600
 P I E S V K N *
 TGATGTTTCCAGGAATTAGTGCACACTGTGATTGTTCTCTCTGTGACAAAACGAAAAATATAAAAAATATCTGCCATTGGTCGATAAAATGTACTGACTGGTGGTGTGATGTCAG 3720
 TCCCTATCTCTATGGTGAGGAACCTCATGCTCAATTAGGAAGACTGACCAATTCGTGACCGGTTTTTGGAGCTTGGCGCAATTAAGCTGCTCTAGA 3818
 Xba I

FIG. 4. Nucleotide and deduced amino acid sequences of *pepXP* and ORF1. Nucleotide residues are numbered in the 5'-to-3' direction, with the first C of CAGCTG of the *PvuII* restriction site being number 1. The restriction sites used for subcloning in the sequencing procedure are indicated. The underlined amino acid sequence was also determined by protein sequence analysis of the purified enzyme. Putative ribosome-binding sites (RBS) and promoter regions are indicated by overlining. T, The divergent arrows above the DNA sequence indicate the putative transcription terminator. SC, Start codons. Asterisks, Stop codons.

enzyme. Since the N-terminal amino acid sequence of the purified enzyme was the same as that predicted from *pepXP*, the enzyme was apparently not subject to processing at the N terminus. Both extracellular (16) and intracellular (41) locations for X-PDAP have been suggested. Careful studies are required to determine the exact location of the enzyme. *pepXP* encodes a protein with a calculated molecular weight of 87,787, a value which agrees well with that of the purified X-PDAP monomer estimated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (16). The putative transcriptional and translational regulatory sequences of the gene closely resemble those reported previously for lactococci (39).

Most of the X-PDAPs isolated from lactic acid bacteria (3, 15, 16, 25, 41), as well as those isolated from other bacteria, yeasts, and eucaryotic sources, have been classified as serine proteinases on the basis of the effect of several substances known to inhibit these types of enzymes. Surprisingly, the sequence of the protein from *L. lactis* subsp. *cremoris* did not show significant similarity to any serine proteinase, including the cell envelope-associated lactococcal proteinase, which was previously characterized as a serine proteinase (18). This result suggests that the X-PDAP from *L. lactis* has a different evolutionary origin.

The primary amino acid sequences of the *pepXP* gene from *L. lactis* subsp. *cremoris* P8-2-47 and the gene from *L. lactis* subsp. *lactis* NCDO 763 were nearly the same. Only seven amino acid substitutions, including two conservative ones, were observed. Also, the putative transcription and translation signals controlling the genes were highly similar. As with the *pepXP* gene from *L. lactis* subsp. *cremoris*, a second, divergently transcribed ORF was present upstream of the gene from *L. lactis* subsp. *lactis* at almost the same distance from the *pepXP* gene (29a).

ORF1 encodes a very hydrophobic protein. Since this protein probably contains at least five regions of sufficient hydrophobicity and length to span the membrane, we suggest that it could be an integral transmembrane protein. The observation that X-PDAP activity in *E. coli* was expressed in the absence of ORF1 (data not shown) argues against a direct role of the ORF1 gene product in the activity of X-PDAP.

X-PDAP has been shown to constitute the major peptidase activity in many lactic acid bacteria (5). It has been suggested that X-PDAP is essential for *L. lactis* subsp. *cremoris* because the enzyme might render the proline from casein available to the cells in the form of dipeptides (5, 36). Dipeptide uptake systems are essential for these bacteria (21, 30, 35), and since they cannot normally take up proline as a free amino acid (35), they may meet their proline requirement by the uptake of proline-containing dipeptides generated by X-PDAP. Whether this is the biological role of X-PDAP remains to be established. The availability of the gene specifying X-PDAP will be helpful in examining the cellular location and role in cell physiology of this enzyme.

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